CYTOCHEMICAL INTERPRETATION OF THE MECHANISM OF PENICILLIN ACTION[1]

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"Among the lower beings, even more than among the higher animals and plant species, life destroys life."

Pasteur and Joubert (1877)

According to current usage, the term antibiotic designates a product of the metabolism of one microörganism that is antagonistic to the continuation of the normal life activities of another microörganism when present even in very low concentrations. Since the early literature on antibiosis has been covered extensively (4, 7, 29, 70, 71, 145, 190), we shall dispense with this phase of the subject.

The penicillins owe their outstanding therapeutic position to the fact that they exert a strong selective action against certain types of bacteria in concentrations far below those required to produce appreciable effects on animal tissues. They may be considered virtually non-toxic. The activity, A, of an antiseptic may be expressed in terms of the maximum volume (in ml) of broth in which 1 gram of compound will inhibit the growth of a test organism; and the toxicity, T, may be expressed as the maximum weight (in g) of some suitable laboratory animal that can be killed by injection of a like quantity of the same compound. The ratio, A/T, may be used as an index of relative activity and toxicity. According to this system, a large number indicates relatively low toxicity and a small number relatively high toxicity in comparison with activity. Using *Staphylococcus aureus* as the test pathogen and adult mice (20 g each, injected intraperitoneally) as test animals, the A/T ratio has been found to be 3 for anemonin, a very toxic compound, 100 for sulfathiazole, and greater than 100,000 for penicillin (28).

The virtual lack of toxicity of penicillin was recognized at an early date when it was shown in 1944 that any toxicity of purified penicillin salts could very likely be attributed to the cation. It was found that the toxicity of the sodium, ammonium, strontium, calcium, magnesium and potassium salts of penicillin ranked in the same order as that of the acetates, which have a non-toxic anion (198).

The only unfavorable physiological effects that have been reported, apart from the urticaria that occasionally develops in sensitive individuals, are delayed clotting of blood (69a, 123), the precipitation of convulsions following intrathecal injection (192), and partial inhibition of phagocytosis (197). With regard to the first of these observations, however, it should be pointed out that a contradictory report has been published, i.e., that penicillin accelerates clotting

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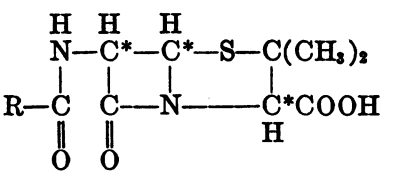
(131). The discrepancy in observations may be due to the widely different concentrations of penicillin that were employed. The other reported untoward effects have been observed only with large doses, far in excess of those administered clinically.

The penicillins are further characterized by the fact that they inhibit susceptible bacteria more effectively both *in vivo* and *in vitro* when the environment is most favorable for growth and by the fact that within wide limits their activity is not affected by the number of bacteria present (16) whereas many other chemotherapeutic agents are less effective if contamination is heavy.

SOME IMPORTANT CHARACTERISTICS OF THE PENICILLINS

The penicillins comprise a family of antibiotics that are biosynthesized by several species of molds, notably penicillia belonging to the notatum-chrysogenum group. More than a dozen penicillins are recognized to occur naturally and from some of these (principally penicillin X) many derivatives have been prepared. However, only five penicillins are commonly encountered, all possessing the same fundamental chemical structure:



They are characterized stereochemically by the three asymmetric carbons (starred in the above formula) which are responsible for the optical rotation of their solutions. The several penicillins differ markedly in their pK values, their distribution between polar and non-polar solvents, and their biological activity *in vitro* and *in vivo* according to the character of the R group (table 1).

Laboratory experiments with animals, and clinical trials and evaluations have shown that the relative effectiveness of the different penicillins *in vivo* depends upon the rate of excretion, degree of protein-binding in the blood, the pathogen involved, and several other factors. In practice, benzyl penicillin (penicillin G) has been found to be the most generally useful (38, 59, 60, 61, 99, 101, 166).

In this paper, following established custom, the term penicillin will be used to designate a salt of benzyl penicillin unless otherwise specified. An average effective therapeutic blood level of penicillin for systemic treatment of many infections is considered to be about 0.075 unit per ml of serum. Since one unit of the pure sodium salt of benzyl penicillin weighs 0.6 microgram, an average effective therapeutic serum level represents a dilution of 1 part in approximately 22 million. For infections due to highly sensitive organisms, serum levels of 0.03 units per ml (1 part penicillin in approximately 55 million) often are adequate. Solutions containing 0.1 unit penicillin per ml, or 1 part in approximately 16.6 million, have been shown to be lethal *in vitro* to cells of average strains of *Staphylococcus aureus*. These concentrations are far below the effective antibacterial concentrations of other antibiotics and of the commonly employed

antiseptics. A comparison of the *in vitro* activity of penicillin with some other antibiotics is shown in table 2. It should be pointed out, however, that the figures are based on agar plate assays and that the results obtained by this technique depend not only on biological phenomena, but also on the physical phenomenon of diffusion. Consequently, since the molecules of some of the compounds may not diffuse as readily as those of others, the exact order of activity for some of the compounds is different in serial dilution tests, although by this test also penicillin ranks first in effectiveness.

As has been pointed out by Dubos (51, 52, 53) and others, the fact that penicillin exerts a strong selective action in relatively low concentrations against many types of bacterial cells without manifesting any appreciable toxicity toward other living structures indicates that it does not owe its action to a general or non-specific protoplasmic poisoning as do many cell poisons such as the halogens, salts of heavy metals, phenols, etc., but suggests rather, that its

TABLE 1

Characteristics of five common penicillins[a]

R GROUP		COMMON NAME	MOL. WT. OF Na SALT	pK	IN VITRO ACTIVITY[b] (UNITS/MG) AGAINST	
Carboxyl residue	Acetamide linkage				*S. aureus*	*B. subtilis*
phenylacetic	benzyl	G	356.2	2.5	1,667	1,667
p-hydroxy-phenyl-acetic	*p*-hydroxy-benzyl	X	372.2	2.5	900	1,500
3-hexenoic (hydro-sorbic)	Δ^2 pentenyl	F	334.2	2.7	1,500	970
caproic	*n*-amyl	dihydro F	335.2	2.7–	?	?
octanoic (caprylic)	*n*-heptyl	K	364.3	5.0	2,300	700

[a] Compiled from data of Schmidt, Ward and Coghill (165), Goodall and Levi (86, 87), Henry and Housewright (95), and unpublished data from the Cutter Laboratories.

[b] Determined by standard 16 hour cylinder-plate assays.

effect is highly specific. Evidence to support this idea is provided by the facts that microörganisms are most susceptible to the action of penicillin when they are in the logarithmic phase of growth and that incorporation in the media of growth stimulating substances enhances the action of penicillin *in vitro* (17, 35, 100, 102, 105, 113, 128, 154). Thus when the cells are dividing most rapidly, and are exhibiting the greatest need for oxygen, they are most sensitive to the action of penicillin. Conversely, conditions (such as low temperature) which decrease the rate of division, tend to decrease the susceptibility of bacteria to the killing power of penicillin (35, 103, 111). In this connection it is interesting to note that strains of staphylococci that are resistant to penicillin have been reported to be characterized by depressed growth rates (72, 75).

Strains of organisms that become resistant to one type of penicillin become correspondingly resistant to the others (62) as would be predicted, since penicillin-fastness depends upon the slowing down of metabolic rates. It should be

noted, however, that acquired "fastness" to penicillin is only temporary. The organisms return, after several generations, to their normal rates of growth and become susceptible again to the action of penicillin (184).

AMORPHOUS VS. CRYSTALLINE PENICILLIN

The earlier reports on the mechanism of penicillin action were based on studies with impure penicillins. Among the impurities that may be found in such

TABLE 2

Parts per million of antibiotics required to produce inhibition zones 20 mm in diameter on standard assay plates seeded with Staphylococcus aureus[a]

ANTIBIOTIC	PPM	AUTHORITY
1. Penicillin	1	Abraham and Chain (1)
2. Helvolic acid	6	Chain *et al.* (34)
3. Mycophenolic acid	31	Clutterbuck and Raistrick (37) Florey *et al.* (73)
4. Protoactinomycin	200	Gardner and Chain (81)
5. Citrinin	62	Tauber *et al.* (180) Heatherington and Raistrick (94)
6. Gliotoxin	31	Weindling (195) Johnson *et al.* (107)
7. Puberolic acid	200	Oxford *et al.* (142)
8. Fumigatin	1,000	Anslow and Raistrick (5) Oxford and Raistrick (140)
9. Spinulosin	4,000	Birkinshaw and Raistrick (21) Anslow and Raistrick (6)
10. Anhydro-3-hydroxy-methylene tetrahydropyrone-2-carboxylic acid[b]	100	Chain *et al.* (33a)
11. Aspergillic acid	1,000	White and Hill (201) Menzel *et al.* (125)
12. Penicillic acid	1,000	Birkinshaw *et al.* (19) Oxford *et al.* (141)
13. Kojic acid	6,666	Birkinshaw *et al.* (18, 20) Jennings and Williams (106)
14. Streptomycin	500	Schatz *et al.* (163) Schenck and Spielman (164)

[a] Calculated and rearranged from data compiled by Heatly and Philpot (93).

[b] Variously named expansin, clavatin, clavacin, claviformin, patulin, penicidin.

preparations are indole-acetic acid (12, 46, 47), phenylacetic acid (13), ortho-hydroxy-phenylacetic acid (67), members of vitamin complexes (162), and other unidentified growth regulating factors (11, 41, 42, 48, 118, 119, 126, 129, 175). It is difficult in many cases, therefore, properly to evaluate the interpretations of the recorded results (153). In view of this fact and since extensive bibliographies of the early work have been published (14, 74, 104, 137), the present discussion is limited to studies with crystalline penicillin or to effects observed in the earlier work and subsequently substantiated with crystalline penicillin.

One current trend in research aims at identifying chemically the various substances that may be present in impure penicillins and which at proper concentrations may shorten the lag period, before the onset of logarithmic growth in cultures of bacteria. This work takes on added significance in view of the recently renewed interest in amorphous (impure) penicillin, some lots of which have been shown to contain a factor or factors that are themselves inactive but that are capable of increasing markedly the effectiveness of pure penicillin *in vitro* (57), and especially interesting, its therapeutic effectiveness *in vivo* (101, 199). Some of these enhancing factors may prove to be subtances that affect the systems regulating the respiratory processes of the pathogen cells, so that the cells become more susceptible to the action of penicillin. It has been shown, for example, that small amounts of cobalt salts, which have been long known to function in oxygen transfer, when appropriately used, increase several fold the bactericidal activity of low concentrations of penicillin *in vitro* (179). Similarly the therapeutic effectiveness of penicillin *in vivo* may be doubled by appropriate use of cobalt (150). The observation that culturing bacteria in the presence of phenylacetates activates the dehydrogenases involved in the oxidation of mandelate and benzaldehyde (178) may afford a basis for elucidating the significance of phenylacetic acid and its derivatives as co-factors in "amorphous" penicillins.

It is recognized that solutions prepared from amorphous penicillin are more stable than are those prepared from crystalline penicillin (45, 132, 173). This has been ascribed to the buffering action, and consequent protective effect, of the impurities present in the amorphous product. It seems likely, however, that more than mere buffering is involved, since it has been shown that addition of small amounts of phosphate markedly stabilizes solutions of penicillin and that this effect probably cannot be accounted for entirely in terms of pH (146, 147). It appears that phosphates or other impurities that are present in amorphous penicillins may bind cations of various metals which otherwise might slowly destroy the activity of the penicillin through esterification or through opening of the thiazolidine ring. It should be pointed out that cations, such as those of heavy metals, etc., that tend to promote inactivation of penicillin during storage may actually enhance its bacteriostatic activity at the time of contact with a living bacterial cell through their ability to function as oxygen carriers (see discussion below).

EVIDENT EFFECTS OF PENICILLIN ON BACTERIA

A. Morphological.—It is well known that bacteria under the influence of bactericidal concentrations of penicillin undergo distortion and swelling and ultimate lysis (3, 10, 15, 51, 68, 69, 80, 82, 114, 117, 128, 129, 148, 172, 174, 181, 182, 194, 196). Microscopical examination of assay plates seeded with different test organisms shows that the cells not only increase in size, but tend to become concatenated. In rod forms such as *Escherichia coli* and *Bacillus subtilis* this becomes manifest by development of elongated mycelium-like structures, and in cocci by the formation of chains of cells. Thus, cells of *S. aureus* under the influence of penicillin tend to form streptococcus-like chains, concomitantly

losing their gram-positiveness (56). This is followed by swelling and abnormal enlargement of the cells. It is interesting to note that this tendency toward concatenation is also shown in streptococci which, under the influence of penicillin, tend to form longer chains than normal (80). It should be pointed out, however, that this response is not specific to penicillin, since it may be induced by sulfonamides (83, 121, 187), by norvaline (155, 189) and very markedly by bacitracin (unpublished data). At sub-bactericidal concentrations, penicillin stimulates metabolism and may act as a "growth factor" in promoting increase of cell size, although at the same time it disharmonizes the processes of cellular enlargement and cellular division.

The modification of the geometrical arrangement of the individuals in the colony may be correlated with a change in the distribution of electrostatic charges at the cell surfaces. Dorfman (49) and Dorfman and Kastorskaya (50) went so far as to attribute the action of penicillin primarily to this effect, and they proposed a method of electrokinetic assay of penicillin based on the increase of ζ values (electrokinetic potentials) of susceptible bacteria under the influence of the antibiotic. The modification of distribution of electrostatic charges on seeded plates exposed to penicillin can be revealed by flooding the plates with suspensions of electropositive or of electronegative particles and by studying the sites of flocculation. The patterns so revealed (55) can be correlated with the patterns revealed by pH indicators and by rH indicators, and the over-all effect can be interpreted in terms of a shift of sulfhydryl (—SH) to disulfide (S—S), of aldehydic COH to carboxylic COOH or ketonic CO, or of enolic COH to ketonic CO.

B. Biochemical Effects.—Gale and Taylor (79) and Gale (77) showed that one of the earliest manifestations of the action of bacteriostatic concentrations of penicillin on *S. aureus* is a blocking of the absorption of the essential metabolite, glutamic acid. Thus their experimental evidence corroborates the earlier postulate (174) that penicillin interferes with the assimilation of essential growth factors by the organism. This effect becomes evident after very short contact of penicillin with the cell, before morphological changes are apparent. The observation that an initial biochemically evident effect of penicillin is inhibition of glutamic acid assimilation appears extremely significant, since glutamic acid is a component of glutathione, the activity of the —SH group of which is known to depend in large measure on the vicinal NH groups (85). Glutathione or similar sulfhydryl bearing proteins are known to act in the aerobic cell as reservoirs of H that are capable of promoting rehydrogenation of the dienol or aldehydic groups that are essential in aerobic respiration. In this connection it is interesting to note that Speck (177) correlated the activity of enzymatic synthesis of glutamine with proteins requiring sulfhydryl groups for full activity. Grossowicz (91) observed that *Neisseria intracellularis* grows readily in a synthetic medium containing glucose (or lactate), glutamine, thiosulfate, and mineral salts, but that it is inhibited by cystine. The dependence of this penicillin-sensitive though gram-negative organism on a reduced form of sulfur (thiosulfate) and its intolerance of a disulfide (cystine) may account for its amenable-

ness to penicillin therapy, and may provide a lead to the cytochemical mode of action of penicillin on organisms, irrespective of their reaction to Gram's stain.

Evidence from cytochemical studies indicates that penicillin exerts its bacteriostatic action by promoting dehydrogenation of —SH groups to S—S more rapidly than the organisms can restore the active sulfhydryl group (55, 56, 148, 149). This hypothesis is in full agreement with the observation that cysteine suppresses the antibacterial activity of penicillin (30–33). Cavallito and coworkers (32, 33) visualized an inactivation of penicillin in a direct stoichiometric relation and expressed some surprise at the ability of cysteine to inactivate "widely different chemical types of antibiotics", although they also recognized the possibility that penicillin action might involve interference with the normal functioning of sulfhydryl groups in bacterial metabolism. It is undoubtedly true that relatively high concentrations of cysteine may chemically inactivate penicillin over a period of hours (32, 36, 96, 133). Chow and McKee (36) showed, for example, that equimolar solutions of penicillin and cysteine react so that positive tests for —SH and NH_2 in the cysteine gradually fade, the penicillin being concomitantly inactivated to penicilloic acid. However, we interpret the fact that cysteine suppresses or reverses the action of penicillin and other unrelated antibiotics (8, 9, 32), of mercurials (40, 66), and of arsenicals (58), when tested by biological assay, as evidence for an indirect action of cysteine. We propose that its effect, especially when present in low concentrations, is exerted not primarily on the antibiotic agent, but rather on the cell of the test organism. That is, *in vivo*, cysteine serves mainly as a source of —SH groups so that the organism has available a reservoir of sulfhydryl groups sufficient to fulfill its requirements, and, therefore, can tolerate concentrations of the antibiotic that would otherwise be toxic.

Mulé (134) having noted that increase in "pressure" (tension?) of hydrogen in broth cultures of *S. aureus* counteracted the bacteriostatic effectiveness of penicillin, postulated that penicillin promotes lethal dehydrogenation in the cell, which may be counteracted by providing hydrogen to activate the respiratory enzymes. He later (135, 136) more specifically inferred that penicillin acts on susceptible organisms by shifting glutathione from the reduced to the oxidized state.

There is good evidence that —SH groups are most readily demonstrated and are most reactive at the time of cellular division (27) and that concomitantly there is an active synthesis of desoxyribonucleic acid derivatives (23, 124, 159, 160, 186, 191). On the basis of this evidence, the —SH groups in organisms exposed to penicillin should be expected to suffer dehydrogenation most readily at the time of cell division. Experimental evidence fulfills this expectation. When exposed to bacteriostatic concentrations of penicillin, sensitive organisms which start to divide fail to complete the division. Thus, penicillin checks the cells in an early stage of division (24, 25, 26, 112), or perhaps even before the first division is completed (35). In this respect, penicillin differs markedly from the sulfonamides which permit several cell divisions to occur. The fact that penicillin and sulfonamides act on bacteria through different mechanisms may

account for the greater effectiveness of combinations of the two drugs than of either independently. This has been interpreted on the basis of selection and inhibition of resistant varieties (109, 109a). Although cells under the influence of penicillin are prevented from yielding two daughter cells, they do evidence excessive increase in size; they swell into diplococcus-like shells that exhibit bipolar staining with vital dyes, the color of which shifts to that indicative of a higher level of oxidation potential (148). Support for the view that there may be a distinct difference between the penicillin-cysteine relation *in vivo* and *in vitro* is suggested by the observations that inactivation of the antibiotic occurs more readily in aqueous solutions of penicillin and cysteine than in the presence of blood (97).

EFFECTS OF BACTERIOSTATIC AND SUB-BACTERIOSTATIC CONCENTRATIONS OF PENICILLIN

Sub-bacteriostatic concentrations of penicillin have been shown to enhance metabolic activity and growth of *S. aureus* (22, 43, 64, 65, 130, 156, 188). This accounts, in part at least, for the narrow ring of enhanced growth that immediately surrounds, or outlines, each zone of inhibition on assay plates. These rings are constant features of assay plates and can be seen on published photographs (65). It has been suggested (55, 149) that they represent regions in which the test organisms, having been subjected to sub-bacteriostatic concentrations of penicillin without subsequent exposure to "static" or "cidal" concentrations, have been stimulated to a state of intense metabolism and growth, characterized by an abnormally high rate of respiration, such as has been ascribed to the "climacteric stage" induced in cells of various tissues by traces of different chemicals. However, other factors may also contribute to the development of a ring of enhanced growth at the boundary between a region of inhibition and the circumjacent uninhibited area of the plates. Cytochemical studies have demonstrated that as cells in the area of inhibition are affected by bacteriostatic concentrations of penicillin, some of their components are liberated into the agar through which they diffuse to regions of the plate in which the concentration of penicillin fails to reach a bacteriostatic level, and it is likely that some of these substances, especially the nucleoproteins, may be absorbed by the bacterial cells outside the zones of inhibition and may serve as metabolites or growth factors. Support for such an hypothesis is provided by the experiments showing that when *S. aureus* was cultured in the presence of low concentrations of penicillin in broth, two waves of growth occurred (2, 24, 25, 200). These results may be interpreted as indicating that when cells of *S. aureus* are suspended in broth containing very low concentrations of penicillin, the most sensitive organisms which are first affected, as they undergo lysis, release into the medium substances which promote a second wave of growth among the more resistant cells. Since lysis of senescent cells normally occurs in cultures of microörganisms, penicillin may be considered as hastening the process of aging. Tulasne and Vendrely (186) have recently stressed the prominent role played by ribonuclease, actively secreted by aging bacteria, in promoting the lysis of the

extra-nuclear content of senescent bacteria, and it is apparent that this process is accelerated in the presence of appropriate concentrations of penicillin (25, 26, 64, 65, 200), making possible, under suitable conditions, post-lytic waves of growth. As early as 1945, Todd (183) had emphasized the possible role of autolytic enzymes in promoting bacteriolysis of cells exposed to penicillin. A number of references in the literature indicate that products liberated by dying microörganisms may serve as growth factors for survivors (39, 110, 122, 139, 193). The observation that in the presence of sufficiently low bacteriostatic concentrations of penicillin, proliferation of *S. aureus* in broth can continue for several hours before clearing of the suspension becomes evident (174) can be interpreted as an indication that penicillin exerts a secondary action by increasing the permeability of the most sensitive microörganisms in the colony so that some of their constituents are released into the medium where they serve to enhance the metabolic activity and to stimulate the growth of more resistant neighboring cells. Such cells would then become more susceptible to penicillin. Thus the course of events in cultures exposed to penicillin might be visualized as analogous to a "chain reaction."

EFFECT OF PENICILLIN ON THE RESPIRATORY SYSTEMS

From the preceding discussion, it is evident that the antibacterial effect of penicillin is more pronounced toward organisms that are growing and respiring vigorously than toward those that are in a resting stage. Culturing cells of *S. aureus* in a protein-free medium, poor in H donors, rapidly brings them to a resting stage, whereupon the respiratory activity falls progressively (Schuler, 167). The rate of fall is not appreciably affected by addition of penicillin (167, fig. 14). Conversely, in nutrient broth, oxygen uptake which is low during the first half hour, increases logarithmically with time, and reaches a maximum about the third hour; this is the time when the density of population of living cells is greatest. Addition of penicillin to the cultures just before or during the logarithmic phase shortens the duration of that phase of respiration and growth, the curtailment being directly related to the concentration of penicillin. Schuler (168, 169) obtained similar curves using streptomycin. Hirsch and Dosdogru (98) duplicated the work of Schuler (167, 170), and it is interesting to note that the corresponding curves are virtually superimposable. In both cases, enhancement of respiration is apparent in organisms exposed to very low concentrations of penicillin, thus providing experimental support for our hypothesis (55, 56, 148, 149) based on interpretation of the color reactions obtained on assay plates treated with suitable dyes and indicators. Schuler further showed that the curve relating evolution of CO_2 to time is also affected by addition of penicillin. The curves for both O_2 consumption and CO_2 evolution decline after the period of maximum activity at about three hours has been passed. The bacteriostatic effect of penicillin is less marked in broth saturated with CO_2 than in aerobic controls, as shown by the fact that the curve for CO_2 evolution falls more abruptly in the aerobic cultures than in those saturated with CO_2 (167, fig. 10). This is completely in accord with the more recent conclusion of Mulé (134) that the

bacteriostatic effectiveness of penicillin is a direct function of O_2 tension in the broth. Schuler (168) later showed that the responses of *E. coli* and of *S. aureus* to penicillin are similar, although the concentrations necessary to induce the typical effects are considerably higher for *E. coli*. Independently, from cytochemical studies, we reached a similar conclusion, i.e., that penicillin affects gram-positive and gram-negative organisms through the same mechanism (149), the principal difference being in the concentration of antibiotic required. From studies with *S. aureus* in the Warburg apparatus (92) it appears that in this organism, as is well known for aerobic cells in general, active O_2 uptake is dependent upon the activity of a cytochrome reducing dehydrogenase-cytochrome-cytochrome-oxidase system in a chain of reactions as portrayed by Grumbach (92). The strong positive reactions given by actively growing *S. aureus* with solutions of tannin, ferricyanide, hematoxylin, and other reagents that form colored iron complexes may be interpreted as further support for the prevalence of iron-protein enzymes in these organisms.

The observation (54, 144, 167, 168) that penicillin inhibits O_2 uptake most effectively in actively growing bacterial cells suggests that penicillin may block

TABLE 3

Diameters of zones of inhibition surrounding penicylinders[a] containing different concentrations of KCN

ORGANISM	PER CENT CONCENTRATION OF KCN		
	0.001	0.01	0.1
S. aureus	14.5 mm	20.4 mm	24.2 mm
E. coli	10.5 mm	12.3 mm	19.0 mm

[a] Outside diameter 8 mm.

the cytochrome-cytochrome-oxidase system. Since this is the portion of the respiratory mechanism that is known to be cyanide sensitive, it seemed of interest to compare the reactions of the penicillin-sensitive *S. aureus* and the penicillin-resistant *E. coli* to cyanide, which has been reported to stimulate oxidation of internal material while inhibiting oxidation of the external substrate (63, 84). This was done by means of a modification of the cylinder-plate technique for assaying penicillin. Seeded plates were incubated for three hours; cylinders were placed thereon and were filled with appropriate solutions of KCN. Then the plates were reincubated for five hours. Following incubation, treatment with Schiff's reagent, the ferricyanide-$FeSO_4$ reagent for Prussian Blue, or other suitable reagents, revealed a contrast between the inhibition zone which remained uncolored and the background which gave a positive color reaction. As shown in table 3, application of a given concentration of KCN resulted in considerably larger zones on plates seeded with *S. aureus* than on those seeded with *E. coli*. The curves relating log diameter of inhibition zone to log dose are homologous, however, just as is true for penicillin.

Similar results were found with NaN_3 toward which, however, the organisms

appeared to be less sensitive. Application of suitable staining techniques previously described (55, 56) showed that the bacteriostatic action of diffusing KCN, like that of penicillin, eventually results in dehydrogenation of sulfhydryl groups of the test organisms. The threshold concentration for antibacterial action of KCN and of NaN_3, as was true for penicillin, appeared to be much lower and more clearly defined for *S. aureus* than for *E. coli*. However, once the threshold concentration has been exceeded, the aerobic respiratory systems become unbalanced, —SH groups are used up faster than they can be restored, and the oxidation-reduction potential rises above a level compatible with life. Thus, agents favoring dehydrogenation of —SH groups should be expected to potentiate the action of penicillin. This may afford an explanation for the recorded observation that dyes in the oxidized state (capable of acting as hydrogen acceptors) enhance the action of penicillin (185). The observation of Quastel and Yates (152) that certain basic dyes of the triphenylmethane series inhibit O_2 uptake in *E. coli* seems pertinent in this connection. Although the full significance of the fact is not apparent at present, it seems worthy of comment that the potentiating action of dyes on penicillin effectiveness is most pronounced against gram-negatives, and that the dyes which are most effective are those which have an affinity for the gram-positive complex. Just as dehydrogenation of —SH groups more rapidly than they are restituted should be expected to enhance the action of penicillin, so binding or otherwise removing —SH groups from active participation in the reversible metabolic processes of the cell should be expected to make penicillin inhibitory at lower concentrations. Evidence that this may occur is found in the fact that bismuth (115, 116) and cobalt (150, 179) in concentrations which are not inhibitory in themselves, do enhance the effectiveness of penicillin action. Likewise, agents that might tend to "unfold" —SH bearing proteins causing the thiol groups to become exposed and more accessible to dehydrogenation should be expected to potentiate the action of penicillin. This may afford an explanation for the synergistic action of detergents such as cetylpryidinium with penicillin (185).

Numerous enzyme systems involved in carbohydrate, nitrogen, and fat metabolism contain essential thiol groups. Simpler metabolites such as cysteine and glutathione (gamma glutamyl-cysteinyl-glycine) also owe many of their biochemical properties to the presence of a thiol group. In fact, it has been suggested that the primary function of glutathione, which is itself a coenzyme for the glyoxalase enzyme system, is the continuous reactivation of —SH cellular enzymes. Many microörganisms require an external source of potential organic thiol groups, and glutathione has been shown to be a growth factor for variant strains of gonococcus (157, 158). The structure of the penicillin molecule can be written in such a way as to show a striking resemblance to glutathione, and it may be suggested that penicillin may compete with glutathione in processes involving H transfer.

The apparent oxidation-reduction potential of reduced glutathione is low enough in the E_0' scale for the threshold mechanism, postulated for the bacteriostatic mechanism of penicillin toward aerobes, to be operative toward anaerobes

also but at a lower level so that the flavin system may be expected to be involved.

EFFECTS OF PENICILLIN INCIDENT TO ALTERED RESPIRATION

Any agent that impairs O_2 uptake correspondingly impairs the ability of the aerobic cell to absorb and to retain ions or molecules. Thus loss of ability to accumulate vital dyes or loss of ability to absorb silver nitrate (anargyrophily) may be used as a measure of inhibition of respiration, and specifically, can be used as a measure of the bacteriostatic effect of penicillin on suitable test organisms. It may be further used to explore the inhibition of a given enzyme, and to correlate such an enzyme with the production of the energy required for the absorption of ions and molecules from the environment, and their accumulation against the concentration gradient. The inhibition of the energy-providing respiratory system may be so great as not only to prevent absorption and retention of materials from the external environment, but to prevent retention by the cells of their normal constituents (56, 148). This is easily demonstrated by treating the test organisms with a solution of silver nitrate or silver chloride. Actively growing organisms manifest argyrophily, that is to say they absorb silver salts which are deposited as vacuolar precipitates of metallic silver. As test organisms undergo the swelling preliminary to lysis, they allow some of the reducing cellular material to leak out of the cells, and to induce an extracellular deposition of silver; therefore, a kind of silver plating occurs around the cells in the pre-lytic state and in their immediate vicinity. This is the basis of a three-hour cylinder-plate assay for penicillin (89). Ultimately, when the oxidation potential has been shifted upwards sufficiently, the cells lose their ability to promote silver deposition; in other words, they reach the stage of anargyrophily (161).

The reducing ability of actively growing *S. aureus* is exhibited similarly in its capacity to reduce nitrate to nitrite: that capacity is inhibited by penicillin in proportion to its concentration, a fact which has been used for quantitative estimation of penicillin (88). Similarly, anaerobes in the presence of penicillin lose their ability to obtain molecular oxygen from appropriate donors. Therefore, proper use of a suitable dye, such as Janus green, as an oxygen donor makes possible quantitative assay of penicillin against anaerobes (151).

When assay plates seeded with aerobic test organisms are flooded with a solution of oxidized Janus green the dye remains oxidized (green) in the inhibition zone, but is reduced almost immediately by the actively growing organisms outside the zones to the pink quinonoid which is intermediate between the fully oxidized (green) and completely reduced (leuco) forms of the dye. Conversely when plates are flooded with a solution of the leuco dye (completely reduced form) a pink color immediately develops in the actively growing colonies outside the zones of inhibition. Thus it appears that in aerobes growing actively on a nearly neutral medium, the potential is above that of the reduced dye but is below that of the oxidized form.

COMPARATIVE EFFECTS OF PENICILLIN UNDER AEROBIC (PETRI PLATES) AND SEMI-ANAEROBIC (BROTH) CONDITIONS

It may be assumed that on penicillin assay plates there is a uniform oxygen tension and that there is a gradient of penicillin concentration which decreases from the cylinders outward. Conversely, it may be assumed that in broth cultures, there is a uniform penicillin concentration in the medium but that there is a gradient of oxygen tension which decreases from the surface downward. An interesting comparison and demonstration of a critical oxidation-reduction potential threshold may be made by applying appropriate redox indicators to cultures on standard assay plates and in serial dilutions.

When assay plates seeded with *S. aureus* and incubated for 16 hours, as in the standard cylinder-plate assay, are flooded with appropriate oxidation-reduction indicators each zone of inhibition is promptly outlined by a ring that is the site of enhanced activity of the cells and that indicates the position of an rH threshold above which the cells are unable to grow (inside of zone) and below which they grow normally (uninhibited background). Likewise, if equal volumes of broth containing different amounts of penicillin are inoculated with equal numbers of organisms of the same age and are incubated until marked bacteriostasis is evident in the tube with the highest concentration of penicillin, addition of a suitable indicator reveals that an rH threshold has been established in each tube and that the depth of this threshold below the surface increases as the concentration of penicillin increases. In fact the depth of the "oxidized" layer is a linear function of the log of the concentration of penicillin in the broth. The more active the dehydrogenases in a given tube, the closer to the surface will a given indicator be reduced to the leuco-compound. The concentration of antibiotic in the broth can, therefore, be expressed in terms of the depth of the layer of the unreduced dye in the upper layer of the suspension.

A comparison of results obtained from *S. aureus* 16-hour assay plates and serial dilution assays treated with a mixture of dimethyl-paraphenylenediamine hydrochloride and thymol is diagrammed in figure 1. Similar results were obtained using Janus green or Indigosol green IB (obtainable from Durand and Hugenin, Basel, Switzerland). The upper row represents the inhibition zones surrounding cylinders containing geometrically increasing concentrations of penicillin on standard 16-hour assay plates. Correspondingly, the lower row represents the depth of the layer of "oxidized" dye in the several tubes. The numerals indicate the concentration of penicillin, in units per ml, in the different cylinders or tubes, which have been spaced on a logarithmic scale in the diagram. The approximately linear relation between response (diameter of zone or depth of "oxidized" layer) and log concentration of penicillin is indicated by the tangent to the inhibition zones on plates, and by the line through the lower edges of the layers of "oxidized" dye in the tube experiments.

Any agent which may serve in oxygen transfer accelerates dehydrogenation in the cells and favors the loss of reducing capacity of the cells. Thus it tends to increase the action of a given concentration of penicillin. For example, co-

baltous salts increase two to four or more times the response *in vitro* to penicillin, and its effectiveness *in vivo* (150, 179).

INFLUENCE OF PENICILLIN ON METABOLISM OF GLUTAMIC ACID

It is interesting to note that Gale (77, 78) and Gale and Taylor (79) observed that failure of cells of *S. aureus* to assimilate glutamic acid was among the first physiologically manifested effects of penicillin action. The significance of this is emphasized by the earlier observation that penicillin-insensitive gram-negative organisms such as *E. coli* are even more insensitive to penicillin in the presence of

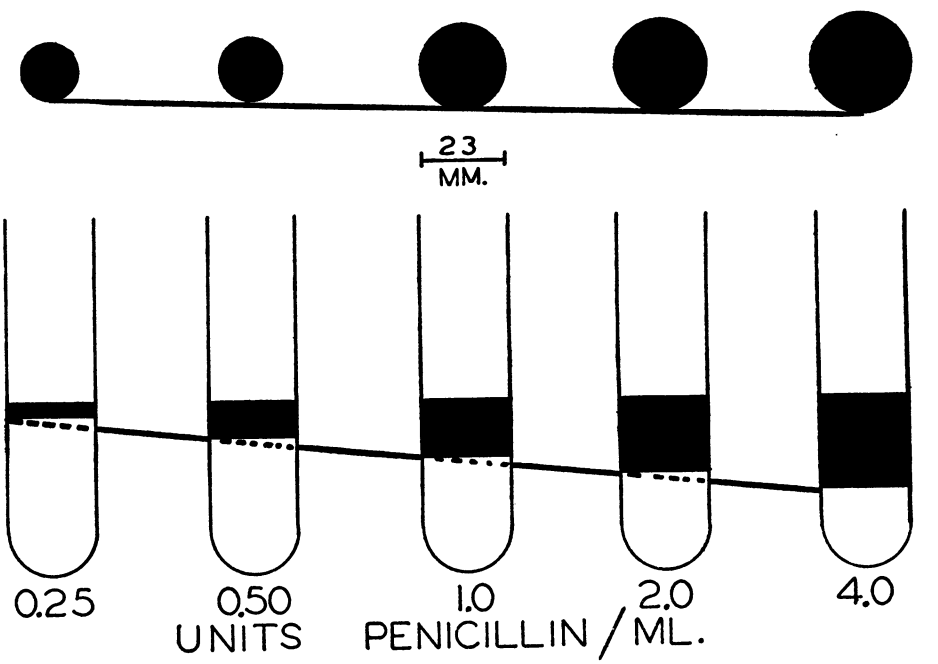


FIG. 1. DIAGRAMMATIC REPRESENTATION OF INHIBITION ZONES ON PENICILLIN ASSAY PLATES AND OF "OXIDZED" LAYERS IN SERIAL DILUTION ASSAYS IN BROTH, FOLLOWING ADDITION OF SUITABLE DYE
(See text for explanation)

glutamic acid (171). The depressing effect of glutamic acid can be blocked or counteracted by the antimetabolite, methionine; in fact, methionine markedly sensitizes gram-negatives toward penicillin (171). At present it is difficult to account for this effect. It should be pointed out, however, that methionine is known to be mostly effective in transmethylation, and the mechanisms of its synergistic effect with penicillin should be studied in this light. The importance of the position of the methyl groups is demonstrated by the fact that *dl*-thiazolidine-4-carboxylic acid in a 1:500 solution is bacteriostatic toward *S. aureus* whereas the 2-dimethyl derivative is inactive even at a concentration of 1:100 (138). Conversely, valine which may be considered to be an analog of the thia-

zolidine ring in the penicillin molecule has been shown in our laboratory to exhibit pronounced inhibition of *S. aureus* on assay plates in concentrations of 1:100,000. Most important among the metabolic processes are transamination, transmethylation, decarboxylation, phosphorylation, and acetylation. Transmethylation has been discussed above in relation to the effect of methionine in association with penicillin. Interference with transamination and decarboxylation might be considered specifically in connection with the inhibition of glutamine metabolism, through interference with the aldehyde or amine pyridoxine derivatives.

Transamination and decarboxylation depend on the phosphorylation of pyridoxal into the active coenzyme (108, 176). It has been shown recently that the inactive apoenzyme of a transaminase system, on addition of pyridoxal phosphate, may reconstitute an active glutaminase or an active tryptophanase (44). Addition of diphosphopyridine nucleotide to the apoenzyme is equally effective. The discussion thus far has been focussed on the interference of penicillin with H transfer. However, phosphorylation, resulting from the proper transfer of PO_4 is as fundamentally important as hydrogenation. It may be suggested that just as supplying the proper H donor may enable microörganisms to survive in the presence of otherwise inhibitory or "cidal" concentrations of penicillin, so supplying suitable phosphorylated compounds (such as nucleic acid) may be similarly effective. This may explain why nucleic acid antagonizes the bacteriostatic action of penicillin (143).

It may be postulated that the different reactions of gram-positive organisms to penicillin as compared with gram-negatives may depend on the different nature of the nucleotides that are present. Examples of differences in the reactivity of nucleotides from different sources with penicillin and other bacteriostatic agents have been reported (14, 90).

Future investigation may show acylation to be also of fundamental importance. Cytochemical evidence from assay plates treated with phenolphthalein phosphate in our laboratory has shown that test organisms outside of the zones of inhibition contain an active alkaline phosphatase, while no trace of phosphatase activity can be demonstrated within the zones of inhibition. Recent studies have emphasized the importance of phosphatases in microörganisms, and in view of the general significance of the energy-rich phosphate bonds in all physiology (120), it appears that further studies may clarify the interrelationship of antibiotics such as penicillin with phosphatases as well as with transaminases. Since penicillin affects cholinesterase in experimental animals (76), it may be suspected of interfering with acetyl-phosphatase activity of microörganisms.

CONCLUSION

The following facts appear to be clearly established by reports in the literature or by our own research:

1. Microörganisms which are metabolizing actively, i.e., those actively absorbing O_2 and evolving CO_2, are most sensitive to the action of penicillin.

2. At a given concentration of penicillin, organisms become more susceptible

to its action as the concentration of O_2 available to the test organisms is greater, or as there is also present some system capable of activating the transfer of O_2.

3. Appropriate sub-bacteriostatic concentrations of penicillin markedly increase the reducing capacity of microörganisms, and this may be interpreted as indicating increased demand for oxygen.

4. At bacteriostatic concentrations, however, penicillin inhibits O_2 uptake and CO_2 release.

5. Thus there results an irreversible dehydrogenation of aldehyde groups and of —SH groups, and consequently the oxidation potential is shifted to a level above that compatible with the proper functioning of the respiratory mechanism. The observations of Prévot (151) suggest that the same threshold mechanism operates for anaerobes as well as for aerobes but at a lower rH value.

Penicillin apparently tends to enhance dehydrogenation in the so-called penicillin-fast organisms, or even in cells of tissues. Ability to survive or even thrive in the presence of penicillin may be correlated with cytochemical ability to restore —SH groups fast enough for the respiratory systems to remain poised within the limits of reversible dehydrogenation and rehydrogenation. An organism may be assumed to be penicillin-sensitive when penicillin stimulates dehydrogenation of its functional sulfhydryl groups faster than they can be restored. Decreasing the rate of H transfer or making extraneous —SH groups available nullifies the effect of penicillin. Increasing the rate at which H_2 becomes accepted by oxygen transporters or donors potentiates the effectiveness of penicillin. Factors which "expose" —SH groups formerly protected in protein molecules, such as detergents, bacteriophage, etc., increase the sensitiveness of the organisms to penicillin.

The sensitive cell is induced into the lethal dehydrogenation of its essential constituents, while releasing into the medium growth-promoting substances which induce nearby cells into a stage of metabolism in which they are penicillin-sensitive. In that sense paraphrasing and adding to the British Medical Journal (October 6, 1945, p. 465) it is difficult to absolve the cell from a charge of collaboration not only in its own death but in that of its relatives.

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